PHENYLTHIOUREA TREATMENT AND BINDING OF RADIOACTIVE IODINE IN THE TADPOLE

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In a study of the distribution of I^{131} after administration to tadpoles, Dent and Hunt (1952) demonstrated not only the expected concentration of this substance in the thyroid gland but also significant accumulations in several other regions. Notable among these were the thymus, the horny teeth, the melanophores of the skin, and the pigmented layer of the retina. The same pattern of distribution was observed in tadpoles of *Hyla, Rana,* and *Bufo* in various stages of development and was not altered by thyroidectomy. It was suggested that, since tyrosine is a precursor of melanin, the localization of I^{131} in pigmented tissues may be attributable to the binding of I^{131} to tyrosine that must be present in those tissues. The hypothesis was also advanced that perhaps the same enzymes that bring about oxidation of tyrosine to melanin are able to facilitate the union of iodine and tyrosine. Gennaro and Clements (1956a, 1956b), studying the binding of I^{131} in the skin of the adult frog, provided evidence to support these views. Our experiments were undertaken as a further investigation of the association of iodine with pigmented tissues and as a further test of the hypothesis cited.

It is known that administration of certain derivatives of thiourea results in an inhibition of melanin formation. This has been demonstrated for mammals (Richter and Clisby, 1941; Dieke, 1947), fishes (Frieders, 1954), and several different amphibians (Lynn and de Marie, 1946; Lynn, 1948; Blackstad, 1949; Millott and Lynn, 1954). In amphibians the results are most striking when these substances are given to embryos or young larvae before any melanophores have appeared. Such individuals do not develop black pigment so long as the treatment is continued. Cessation of treatment is followed by rapid melanogenesis. Since it has been demonstrated (Bernheim and Bernheim, 1942; Paschkis, Cantarow, Hart and Rakoff, 1944; Dubois and Erway, 1946) that thiourea derivatives inhibit tyrosinase activity *in vitro,* it is assumed that their role in preventing melanin formation in frog embryos is the inhibition of tryosinase activity. In our experiments, larvae of several different ages were treated with one of these tyrosinase inhibitors, phenylthiourea, to obtain unpigmented tadpoles or tadpoles with reduced pigmentation. The pattern of iodine uptake in these animals was compared with that in untreated controls at various times after the beginning of treatment.

Materials and Methods

The animals used for this experiment were tadpoles of *Hyla versicolor versicolor* LeConte hatched from eggs collected in a small temporary pool near Oak Ridge,

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Tennessee. A few hours after hatching, the larvae were distributed in groups of fifteen in finger bowls, each containing 200 ml. of spring water. On the first, fourth, and fifteenth days after hatching, experimental series were established in this way: Larvae were transferred to six finger bowls, fifteen larvae to each bowl; two contained 200 ml. of 0.01% phenylthiourea in spring water, two contained 200 ml. of 0.005% phenylthiourea in spring water, and two contained spring water alone (controls). The tadpoles, kept at laboratory temperatures (21°–23° C.), were fed crumbled pellets of Purina rat chow and, occasionally, a strained beef-and-liver soup prepared for infants. The culture fluids were changed daily and there was no mortality. At four-day intervals three animals from each experimental and control group were selected at random and examined under the binocular microscope. Records were kept of the gross changes in pigmentation and of the developmental stage reached. The system of staging devised by Taylor and Kollros (1946) for *Rana pipiens* was used and adapted with minor variations for *Hyla versicolor*.

At three ages (4, 24, and 29 days after hatching), animals were removed from the experimental and control series and used in the preparation of autoradiograms.[4] The procedure was as follows: Five larvae from each bowl were put in 50 ml. of a solution of one part per million of stable sodium iodide [5] and enough radioiodine to give an activity of one μc./ml. at the beginning of the immersion period. For each experimental group, one set of five animals was put in a radioiodine solution made up in spring water and another was put in a radioiodine solution made up in the same phenylthiourea solution in which the animals had been raised. After 24 hours in the radioiodine solution, the larvae were passed through two baths of spring water and left in a third bath of spring water, or the appropriate phenylthiourea solution, for another 24 hours. All were then fixed in a 1:1 mixture of Bouin's fluid and Cellosolve. After 8 hours' fixation, they were dehydrated in Cellosolve, embedded in paraffin, and sectioned at 10 micra. The mounted and dried sections were passed through two changes of xylol, transferred to absolute alcohol, and then dipped in a 1.0% solution of collodion in ether-alcohol, and dried. The slides were attached by stationer's binder clips against the emulsion of Eastman medium contrast lantern slide plates in the darkroom and left for 8 days. Finally, the lantern slide plates were developed and the sections themselves were stained with Harris' haematoxylin and Ponceau de xylidine-orange II (Gray, 1952).

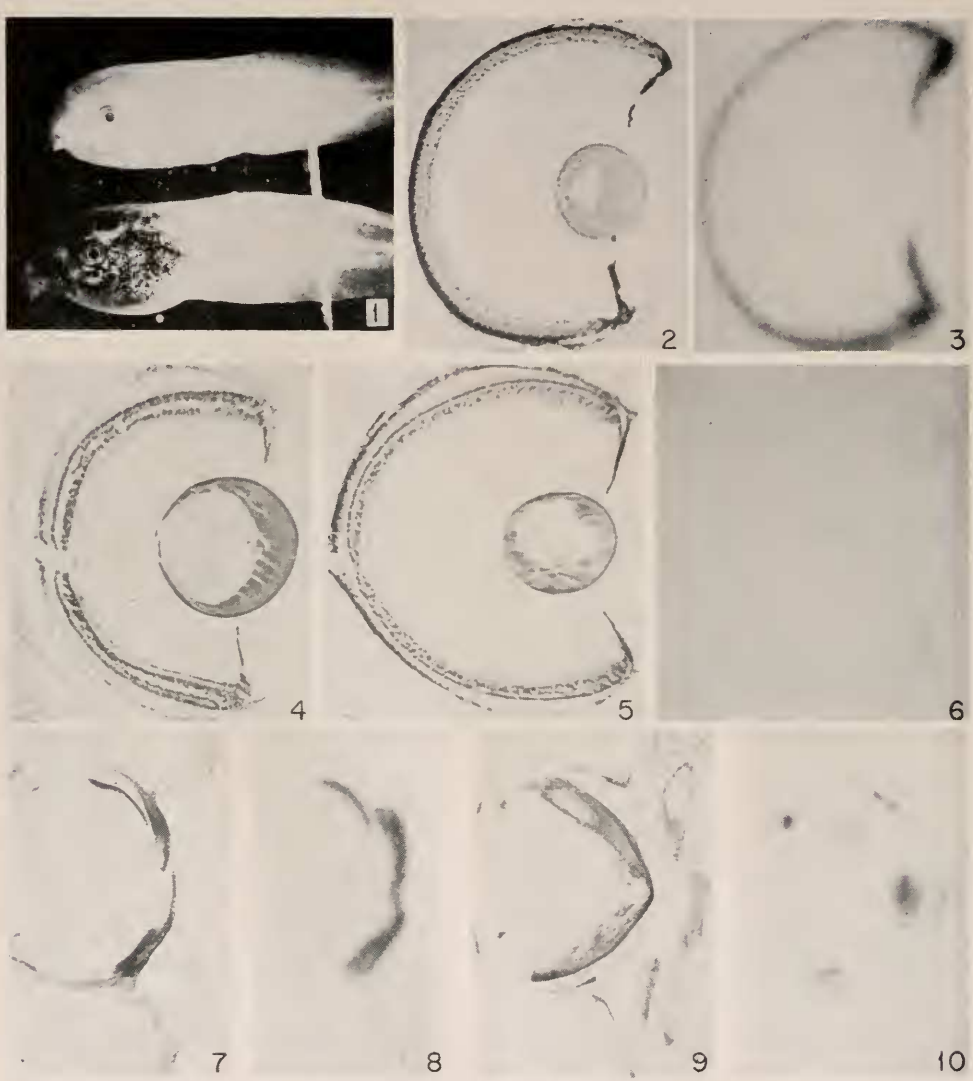
Results

1. *Gross effects of treatment with phenylthiourea*

During ovogenesis (Kemp, 1953) melanin granules are laid down in the cortical region of most anuran ova. Superficially, the pigmented area extends from the animal pole to the presumptive germ ring. These granules are retained within

[4] Joftes and Warren (1956) have recommended the substitution of the term "radioautogram" for "autoradiogram." It is felt that since the semantic basis offered by Joftes and Warren for the use of the former term does not appear to be much stronger than the etymological basis presented by Boyd (1955) for the use of the latter and since the latter term has become well established, its use should be continued.

[5] There is some danger of introducing errors by use of carrier-free isotopes because of their tendency to adhere to glassware. The stable NaI was added with the view of eliminating that effect.



All sections shown are from 26-day-old larvae of *Hyla versicolor* that had been immersed in a solution of one μc of I^{131}/ml. for 24 hours and fixed 24 hours after removal from the solution.

FIGURE 1. Photograph of living tadpoles 12 days old. Upper animal raised in 0.01% phenylthiourea; lower animal raised in spring water.

FIGURE 2. Section through the eye of a control tadpole.

FIGURE 3. Autoradiogram prepared from the section shown in Figure 2.

FIGURE 4. Section through the eye of a tadpole kept in 0.01% phenylthiourea continuously.

FIGURE 5. Section through the eye of a tadpole kept in 0.01% phenylthiourea for 24 days, then in spring water for two days.

FIGURE 6. Autoradiogram of the section shown in Figure 5.

FIGURE 7. Section through the mouth of a control tadpole showing the horny teeth.

FIGURE 8. Autoradiogram of the section shown in Figure 7.

the presumptive ectoderm and mesoderm but disappear as melanophores begin to differentiate and form melanin.

As was anticipated on the basis of previous experiments with other amphibians, the larvae that had been put in phenylthiourea solutions within a few hours after hatching stopped forming melanin and after only 24 hours were noticeably paler than controls. After 48 hours, and at all later stages, the experimental animals were completely unpigmented, the embryonic pigmentation having disappeared (Fig. 1). Larvae in which treatment was delayed until four days after hatching had already developed much melanin in both skin and eyes and exhibited no difference from the controls for at least three days. After this they gradually paled, however, and by the tenth day of treatment their skin was without pigment. Pigmentation of the eyes was lost much more slowly and even when the experiment was terminated (51 days after hatching), the eyes of these animals still showed some pigment, though far less than those of controls. Larvae that were first put into phenylthiourea solutions 15 days after hatching showed no blanching of the skin until near the end of the experiment, and the pigmentation of the eyes never became grossly different from that of controls. Examination of the living animals under the binocular microscope and later study of the sectioned material revealed no significant pigmentary difference between animals treated with 0.01% phenylthiourea and those treated with 0.005%. It appears that both concentrations are fully effective in inhibiting melanin formation.

Since phenylthiourea is one of the well-known thyroid-inhibiting drugs, the experimental animals not only differed from the controls in degree of pigmentation but also in their failure to exhibit definitive metamorphic changes. No significant differences in development were noted for the first 20 days of the experiment. At this time both treated and untreated tadpoles were in late limb bud stages (Stages IV and V). Later, however, the experimental animals showed definite inhibition in development. By 24 days the controls were in Stages VI and VII whereas the tadpoles in phenylthiourea solutions remained at Stages IV and V. The controls continued to differentiate steadily, most specimens reaching Stage IX by the 28th day, Stage XV by the 40th day, and late metamorphic stages (Stages XVIII to XXV) by the 48th day. As is usual with anuran larvae, there was a considerable variation in developmental rate among the controls, a few specimens being retarded and others exceptionally advanced. Thus, although forelimb emergence occurred in one control 45 days after hatching, and had occurred in more than half the surviving controls by 51 days, at this latter time there was still one animal at Stage VI and several at Stage XIII. Among the tadpoles placed in 0.01% phenylthiourea, either immediately after hatching or 4 or 15 days later, none advanced beyond Stage VI and most remained at Stage IV or Stage V. Tadpoles raised in 0.005% phenylthiourea advanced somewhat beyond those in the higher concentration; most of those kept to the end of the experiment reached Stage VI or Stage VII and a few specimens differentiated to Stage VIII. There is thus some indication that the lower concentration is not fully effective in inhibiting thyroid activity.

FIGURE 9. Section through the mouth of a tadpole treated continuously with 0.01% phenylthiourea.

FIGURE 10. Autoradiogram of the section shown in Figure 9.

As noted previously, the larvae used for the preparation of autoradiograms were put in a radioiodine solution for 24 hours, and this was followed by another 24-hour period without radioiodine, which allowed for elimination of excess iodine before fixation of the tadpoles. Autoradiograms were made from two sets of phenylthiourea-treated animals, one in which the phenylthiourea treatment was continued until fixation and one in which the radioiodine solution and the subsequent 24-hour bath were without phenylthiourea. The latter thus had a 48-hour period of recovery from phenylthiourea treatment immediately preceding fixation. It has been demonstrated that in amphibian embryos melanin reappears very rapidly after phenylthiourea treatment has stopped (Millott and Lynn, 1954). In the present experiments the animals put in radioiodine solutions containing no phenylthiourea showed a well-defined darkening of the eyes within 12 hours; and at 24 hours, when they were removed from the radioiodine solutions, a scattering of pigmented melanophores was also visible in the skin. Larvae kept in phenylthiourea solutions throughout, of course, showed no such pigmentary change.

2. *Effects of phenylthiourea treatment on radioiodine binding in the pigmented epithelium of the retina*

The first group tested for radioiodine binding consisted of animals on which the phenylthiourea treatment started on the day of hatching and continued for four days only. The second and third groups tested included animals on which treatment was begun on the day of hatching and some in which treatment was started later (4 and 15 days after hatching). Since the results were similar in all three groups, detailed consideration will be given for only one, the second experimental group, which was given radioiodine on the 24th day after hatching and contained animals under treatment for 24, 20, and 9 days.

Photomicrographs and corresponding autoradiograms of the eye region in typical animals of this group are shown in Figures 2–6. Figures 2 and 4 illustrate the conditions found in control and experimental animals of the series in which phenylthiourea treatment was started at hatching. The pigmented epithelium of the retina is quite dark in the controls but is entirely without melanin in the animals given continuous phenylthiourea treatment. The autoradiogram prepared from the control (Fig. 3) shows that there was a marked concentration of radioiodine in the pigment epithelium. On the other hand, an autoradiogram prepared from the eye shown in Figure 4 was entirely blank and is not shown. It has been pointed out that some melanin formed in the animals allowed a recovery period from treatment with phenylthiourea. Plates prepared from these specimens show faint but definite autoradiograms, indicating that some binding of radioiodine occurred both in the skin and in the retina (Figs. 5, 6). On the basis of these results it might be concluded that the binding of radioiodine depends on the presence of melanin and varies directly in amount with the amount of melanin present. However, study of the larvae in which phenylthiourea treatment was initiated at later ages reveals that this is not the case. It will be remembered that pigment was lost from the eyes only very slowly in the series started at 4 days and not at all, so far as could be seen externally, in the series started at 15 days. Thus these latter tadpoles, even though under continuous treatment with phenylthiourea, still had much melanin in the pigment epithelium. Nevertheless the autoradiograms

prepared from this series exhibited the same variations as those previously described. Control animals showed high radioiodine level, animals treated continuously with phenylthiourea showed no radioiodine binding, those treated with phenylthiourea and then allowed two days' recovery showed a very low radioiodine level. Yet the degree of pigmentation of the members of all groups was about the same. It is therefore clear that the binding of radioiodine does not depend on the amount of formed melanin present.

3. *Effects of phenylthiourea treatment on radioiodine binding by the horny teeth*

In all larvae given radioiodine a significant localization was found in the horny teeth. The 4-day series (fixed at 6 days after hatching) shows relatively little cornification of the teeth. Nevertheless the autoradiograms indicate that radioiodine was bound in these structures, not only in the controls but also, and apparently to the same extent, in the phenylthiourea-treated animals. The 24- and 39-day series both exhibit extensive cornification of the teeth, and in these there is an indication that phenylthiourea did lessen the binding of radioiodine without, however, completely halting it. Photomicrographs and corresponding autoradiograms of the teeth in control and experimental animals of the 24-day series will serve to illustrate this inhibitory effect (Figs. 7–10). It was found that whereas the autoradiograms of control tadpoles (Fig. 8) and those of treated animals allowed a 48-hour recovery period show equally dense spots, representing the teeth, autoradiograms of larvae subjected to continuous treatment with phenylthiourea show definite but much less intense darkening (Fig. 10). This result was consistent in all specimens of the older series.

4. *Effects of phenylthiourea treatment on the thyroid and thymus*

Although these experiments were not primarily concerned with the thyroid gland, some observations on the thyroid response are of interest. In most of the control larvae of the 4-day series the thyroid proved to be at a stage of early follicle formation. Only one or two follicles were present in each thyroid, the rest of the gland consisting of irregular cords of cells. The formed follicles had a cuboidal epithelium that still retained some of the pigmentation characteristic of the anuran thyroid rudiment. The lumina were very small and contained no stained colloid. Two of the five control specimens had no organized follicles at all, the entire thyroid being composed of clumps and cords of epithelial cells. In the experimental animals of this series, there were some with glands lacking organized follicles; these showed no histological differences from the two controls just mentioned. On the other hand, the phenylthiourea-treated larvae in which follicles had appeared showed a sharp contrast to controls in that the follicles were larger, had a flattened epithelium, and contained a relatively large amount of homogeneous basophilic colloid. The autoradiograms of the control thyroids and those allowed 48 hours' recovery from phenylthiourea showed evidence of binding of radioiodine, whereas the animals treated continuously with phenylthiourea did not. It is noteworthy that the thyroids of all 5 of the controls and of all 5 of the recovery series produced clear autoradiograms but in some of them no organized follicles were yet present. The effect of phenylthiourea treatment on thyroid

physiology is thus detectable histologically as soon as follicles are formed and physiologically even before follicles are formed.

The thyroids of controls fixed at 26 days after hatching showed a quite uniform appearance. The follicular epithelium was cuboidal and the colloid acidophilic, usually with some chromophobe droplets. Experimental animals differed consistently in having many more chromophobe droplets and slightly higher epithelium. The different lengths of treatment with phenylthiourea resulted in no histologically observable differences. There was also no significant difference in the appearance of the thyroids of animals treated continuously and those allowed two days' recovery before fixation. Autoradiograms prepared from the sections of this series showed a high level of radioiodine in the control thyroids, a very low level in the thyroids of larvae treated continuously with phenylthiourea, and a high level, apparently as high as that of controls, in those of larvae removed from phenylthiourea at the time of their exposure to radioiodine solution.

The controls of the series fixed at 41 days after hatching were all in metamorphic stages and, as would be expected, their thyroids gave indications of high activity. The epithelium tended to be columnar and chromophobe droplets were abundant. The experimental animals, on the other hand, had enlarged follicles with a markedly flattened epithelium and much homogeneous colloid. The autoradiograms of this series are similar to those for the 24-day series.

The concentration of radioiodine in the thymus gland observed by Dent and Hunt (1952) was confirmed in these experiments and proved to be affected by phenylthiourea treatment in exactly the same way as is radioiodine concentration in the thyroid. Autoradiograms made from control tadpoles of the 24-day series or from tadpoles allowed a recovery period show high concentration of radioactivity in the thymus; those made from tadpoles under continuous treatment with phenylthiourea show no radioactivity in this region.

Discussion

In these experiments the administration of phenylthiourea to early larvae of *Hyla versicolor versicolor* resulted in the production of completely unpigmented tadpoles. Both concentrations tested (0.01 and 0.005%) proved equally effective and neither gave any indications of toxicity. The gradual blanching of the skin produced by treatment with phenylthiourea has sometimes been referred to as a depigmentation effect. It is probable, however, that the drug has no effect on any pigment already present when treatment is begun but acts entirely by preventing the formation of new pigment. The results of our experiments are in accord with this view for, as has been pointed out, the blanching of the skin (and of the eyes) occurred rapidly in larvae treated immediately after hatching, more slowly in those in which treatment was delayed until 4 days after hatching, and very slowly indeed in those in which treatment was begun 15 days after hatching. It must be assumed that in all these animals the phenylthiourea treatment effectively blocked melanogenesis and that the rate of "depigmentation" depended on the rate of loss of the melanin already present. In fact, it would appear that this rate of blanching after treatment with phenylthiourea should furnish an indication of the normal rate of metabolic turnover of melanin at various ages. Our results indicate that turnover is rapid at early ages but much slower in older animals. In fact, it seems

likely that some of the formed melanin persists indefinitely after a certain age is reached.

These experiments also demonstrate that phenylthiourea affects the binding of radioiodine by the tapetum nigrum. Only the untreated animals show a significant concentration of I^{131} by this structure. Tadpoles given phenylthiourea and then removed from the solution exhibit an ability to bind iodine within the first 24 hours after cessation of treatment. In all experiments, however, the autoradiograms, though they do not give quantitative information, indicate clearly that I^{131} is not taken up by the pigmented epithelium of the eye in direct proportion to the amount of melanin present. Tadpoles for which phenylthiourea treatment is begun at 15 days after hatching retain much pigment in the eye yet show no tendency to bind I^{131}. This indicates that the binding of iodine in the pigmented epithelium of the eye (and doubtless in chromatophores as well) takes place only while melanin is actually being formed and is dependent on some enzymatic activity that is inhibited by phenylthiourea. Since this substance is known to inhibit tyrosinase activity *in vitro* and since tyrosine must be present where melanogenesis is going on, it is natural to suspect that tyrosinase is the enzyme involved. These views are supported by the findings of Gennaro and Clements, who extracted radioactive mono- and diiodotyrosine from discs of frog skin that had been incubated in Ringer solution containing I^{131} and from the skins of intact frogs injected with I^{131} (1956a). They also showed that pretreatment of the discs with thiourea decreased the degree of incorporation of I^{131} in the melanized areas (1956b). According to the concept outlined, tyrosinase would be active in pigment-forming tissues both in the oxidation of tyrosine to melanin and in the oxidation of iodide to iodine to permit the production of mono- and diiodotyrosine and possibly iodinated proteins. Inhibition of tyrosinase activity would thus be expected to result simultaneously in cessation of melanogenesis and failure to bind I^{131}. Whether these findings can be directly related to the goitrogenic effects of phenylthiourea is not certain. The mechanism by which iodination of tyrosine occurs in the thyroid is not well understood (Roche and Michel, 1955). There is no evidence of the presence of tyrosinase in the mammalian thyroid (Pitt-Rivers, 1950). Fawcett and Kirkwood (1954) have hypothecated a "tyrosine iodinase" as a catalyst for the process. There are many varieties of tyrosinase (Lerner and Fitzpatrick, 1950), however, and it may be that amphibian tyrosinase has a special property of oxidizing iodine or, on the other hand, that our experiments may offer the key to a better understanding of iodination of tyrosine in the thyroid itself.

The accumulation of iodine in the horny teeth of larval anurans was first reported by Dent and Hunt (1952). Association of iodine with similar hard structures is known to occur in a number of invertebrates. Noteworthy examples are the hypodermis of Drosophila larvae (Wheeler, 1950), the setae and pharyngeal teeth of polychaetes (Swan, 1950), and the exoskeleton of Daphnia (Gorbman, Clements and O'Brien, 1954). Gorbman (1955) has discussed this matter in some detail and notes that in all these cases the localization of radioiodine is in scleroprotein. The present experiments indicate that phenylthiourea treatment, if given over a sufficiently long period, has an inhibitory effect on the binding of radioiodine here although it does not completely prevent it.

The effects of derivatives of thiourea on the functioning of the thyroid gland have been widely studied in mammals and in several amphibians. The histological

changes seen in the thyroids of the animals studied in our experiments are in agreement with those previously reported and need not be discussed. The autoradiographic analysis of the thyroids of the control and experimental animals also gave results that would be expected on the basis of what is known of the effects of this drug. Larvae under continuous treatment with phenylthiourea showed an extremely low ability to bind I^{131}. However, treated larvae recovered this ability very rapidly after treatment was discontinued. This rapid rate of recovery is in contrast with the slower rate observed in the pigmented regions and may be indicative of a difference in the mechanism of iodine binding.

The basis for the accumulation of radioiodine by the thymus gland is not known. It was first reported by Dent and Hunt (1952), and it is clearly demonstrated in our material. It is completely inhibited in animals under continuous treatment with phenylthiourea but cessation of treatment is followed by prompt recovery of the animal's ability to concentrate iodine. It appears, then, that the binding of iodine in the thymus is more closely related to that process as it occurs in the thyroid than as it occurs in the melanophore.

Earlier observations on various vertebrates (see citations in Dent and Hunt, 1954) have all been to the effect that the onset of iodine accumulation by the thyroid does not occur until discrete follicles make their appearance. It is of some interest, then, that in the animals studied here iodine concentration began while the cells of the thyroid rudiment were still arranged in cords at four days after hatching. Moreover, such animals, when given continuous phenylthiourea treatment, showed no ability to concentrate I^{131}. Thus the iodine-concentrating activity of thyroid tissue, and also the ability of phenylthiourea to inhibit this activity, are evidenced well before the appearance of follicles or colloid.

Summary

1. Larvae of *Hyla versicolor* were immersed in solutions of phenylthiourea at 0, 4, and 15 days after hatching. At 4, 24, and 39 days after hatching, I^{131} was administered and contact autoradiograms were prepared from serially sectioned representative specimens.

2. The tadpoles treated with phenylthiourea from the time of hatching became completely unpigmented. The blanching of the second series was slower and never complete. The third series became very little lighter during the course of the investigation. This indicates that the metabolic turnover of melanin goes on at a decreasing rate as the larvae increase in age.

3. From the autoradiograms, evidence was obtained to confirm earlier findings of the binding of iodine in pigmented areas, to show that the binding is apparently not associated with formed melanin, and to support the view that the same enzyme or enzymes that catalyze melanogenesis can catalyze the binding of iodine (presumably with tyrosine).

4. The accumulation of iodine by the horny teeth was inhibited to some degree by phenylthiourea treatment.

5. Accumulation of radioiodine by the thymus gland was confirmed and was found to be completely inhibited by phenylthiourea treatment.

6. The thyroid rudiment acquires the facility for concentrating iodine even before follicle formation begins, and at that time it also responds to the inhibitory action of phenylthiourea.

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